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The ubiquitin-proteasome system is necessary for long-term synaptic depression in *Aplysia*

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Abstract

The neuropeptide Phe-Met-Arg-Phe-NH₂ (FMRFa) can induce transcription-dependent long-term synaptic depression (LTD) in *Aplysia* sensorimotor synapses. We investigated the role of the ubiquitin-proteasome system and the regulation of one of its components, ubiquitin C-terminal hydrolase (ap-uch), in LTD. LTD was sensitive to presynaptic inhibition of the proteasome and was associated with upregulation of ap-uch mRNA and protein. This upregulation appeared to be mediated by CREB2, which is generally regarded as a transcription repressor. Binding of CREB2 to the promoter region of *ap-uch* was accompanied by histone hyperacetylation, suggesting that CREB2 can not only inhibit but also promote gene expression. CREB2 was phosphorylated after FMRFa, and blocking phospho-CREB2 blocked LTD. In addition to changes in the expression of ap-uch, the synaptic vesicle-associated protein synapsin was downregulated in LTD in a proteasome-dependent manner. These results suggest that proteasome-mediated protein degradation is engaged in LTD and that CREB2 may act as a transcription activator under certain conditions.

Keywords

p38 kinase; lactacystin; ubiquitin C-terminal hydrolase; CREB2; histone acetylation; synapsin

Long-term synaptic plasticity, which is a mechanism for long-term memory, requires synthesis of new RNA and protein (Pittenger and Kandel, 2003). This requirement holds true regardless of the direction of the plasticity, potentiation or depression (Stanton and Sarvey, 1984; Frey et al., 1988; Montarolo et al., 1988; Bailey et al., 1992; Nguyen et al., 1994; Linden, 1996; Ahn et al., 1999). In *Aplysia*, long-term depression (LTD), a transcription- and translation-dependent form of synaptic plasticity, can be induced by application of the endogenous tetrapeptide Phe-Met-Arg-Phe-NH₂ (FMRFa; Montarolo et al., 1988; Bailey et al., 1992). Knowledge about the molecular targets of FMRFa during LTD is limited (Guan et al., 2002). It is known, however, that FMRFa leads to activation of p38 MAPK (mitogen-activated protein kinase), which is necessary for LTD (Guan et al., 2003; Fioravante et al., 2006). It is also known that FMRFa promotes structural changes, including loss of presynaptic varicosities and retraction of neurites (Schacher and Montarolo, 1991). The structural rearrangements could depend on selective protein degradation; however, this hypothesis has not been previously investigated.

Recently, a role for protein degradation in long-term plasticity has emerged (Bingol and Schuman, 2005). A central pathway through which selective proteins are degraded involves

the ubiquitin-proteasome system (UPS) (Hegde and DiAntonio, 2002). In this system, target proteins are tagged by the attachment of ubiquitin molecules, which serve as a recognition signal for degradation by the proteasome system. This process is highly regulated by many enzymes, including deubiquitinating enzymes. By removing ubiquitin from proteins prior to their degradation, deubiquitinating enzymes could promote recycling of ubiquitin and facilitate processing through the UPS (Wing, 2003). Early evidence for the importance of the UPS in synaptic plasticity came from studies of long-term facilitation (LTF) in *Aplysia* (Chain et al., 1999b), in which injection of UPS inhibitors blocked LTF. Subsequent studies in mammalian systems corroborated the requirement of the UPS in plasticity and memory (Lopez-Salon et al., 2001; Colledge et al., 2003; Wood et al., 2005). However, little is known about the role of UPS in LTD in *Aplysia*.

A key component of the UPS that is involved in the regulation of LTF in *Aplysia* is ubiquitin C-terminal hydrolase (*ap-uch*) (Hegde et al., 1997). Synthesis of *ap-uch*, a deubiquitinating enzyme, is increased during LTF and is necessary for consolidation of LTF (Hegde et al., 1997). The promoter region of *ap-uch* was recently cloned and a cAMP response element (CRE) was identified (Mohamed et al., 2005). The presence of a CRE raised the possibility that transcription factors of the CREB family may regulate expression of *ap-uch*.

The gene family of CREB transcription factors is important for long-term synaptic plasticity in both vertebrates and invertebrates (Lonze and Ginty, 2002). In *Aplysia*, two CREB genes have been identified: *creb1* and *creb2*. The *creb1* gene encodes two alternatively spliced mRNAs that are translated into three proteins, CREB1a, CREB1b and CREB1c (Bartsch et al., 1998). CREB1a, often referred to simply as CREB1, is homologous to mammalian CREB and is a transcription activator necessary for LTF (Dash et al., 1990). The *creb2* gene gives rise to CREB2 protein, which is homologous to vertebrate CREB2 and ATF4 (Bartsch et al., 1995) and is regarded as a transcriptional repressor that may pose inhibitory constraints on memory formation (Abel et al., 1998; Guan et al., 2002). This constraint can be regulated by phosphorylation (Guan et al., 2002; 2003).

In this study we investigated mechanisms underlying FMRFa-induced LTD. We report that LTD was sensitive to presynaptic disruption of proteasomal function and correlated with increased levels of protein ubiquitination. We also identified *ap-uch* as a target gene that is upregulated during LTD at least partly in a p38 MAPK-dependent manner. This upregulation was probably mediated by CREB2, which is traditionally regarded as a transcription repressor. CREB2 was phosphorylated during LTD at a putative MAPK site and this phosphorylation of CREB2 was necessary for LTD. Finally, we found that synapsin, a synaptic vesicle protein implicated in synapse formation and stability, was downregulated during LTD in a proteasome-dependent manner.

MATERIALS AND METHODS

Treatments

Ganglia and/or co-cultures were treated with FMRFa (American Peptide Inc., Sunnyvale, CA; 10 μ M) or artificial seawater (for composition see Antzoulatos et al., 2003) using two protocols, as indicated: five 5-min pulses at an inter-pulse interval of 20 min (approximately 85 min in total); or 90-min continuous application. The p38 MAPK inhibitor SB203580 (EMD Chemicals, Gibbstown, NJ; 20 μ M) was applied 30 min prior to FMRFa treatment and was present throughout FMRFa exposure.

Electrophysiology

Sensory-motor co-cultures of pleural sensory neurons and L7 motor neurons were prepared as described previously (Angers et al., 2002). Excitatory postsynaptic potentials were recorded from motor neurons with 10-15 M Ω sharp electrodes filled with 3 M potassium acetate. Stimulation of presynaptic sensory neurons was performed extracellularly using a blunt, patch-type electrode filled with 50% modified L15 medium-50% ASW. Data acquisition was performed using pClamp (Axon Instruments). For statistical analysis, the amplitude of the EPSP in mV was measured during the pre-test and 24 h after exposure to FMRFa or vehicle (post-test) and the post/pre ratio was formed.

Aplysia CREB2 antibodies

The polyclonal anti-phospho-CREB2 and anti-CREB2 antibodies used in this study have been described previously (Mohamed et al., 2005). Briefly, both antibodies were raised by a commercial vendor (Genemed Synthesis, Inc., South San Francisco, CA) against a hybrid *Aplysia* CREB2 peptide with sequence SPPDSPEQGPSSPET. This peptide was constructed to juxtapose the sequences immediately surrounding two putative MAPK phosphorylation sites on *Aplysia* CREB2 (underlined). The phosphorylated version of the peptide, with phosphate groups chemically added on Ser¹⁵² (1st underline) and Ser²³⁷ (2nd underline), was used to raise the anti-phospho-CREB2 antibody, whereas the unphosphorylated version of the peptide was used to raise the anti-CREB2 antibody. Both antibodies were purified through columns with the corresponding antigen. The anti-phospho-CREB2 antibody was further purified through a second affinity column with the unphosphorylated peptide.

Antibody injections

Anti-phospho-CREB2 antibody or purified rabbit IgG (both at 0.43 mg/ml) in injection buffer (100 mM KCl, 0.1% 10 kDa dextran-Alexa 488) was pressure-injected in the cytoplasm of sensory neurons. The efficiency of injections was monitored with a fluorescence microscope. Purified IgG, as opposed to pre-immune serum, was used in this experiment because the anti-phospho-CREB2 antibody was affinity-purified. Purified IgG has been used as control for affinity-purified antibodies in various systems (e.g., Pepperkok et al., 1994; Buffo et al., 2000; Iriyama et al., 2007).

qRT-PCR analysis of ap-uch mRNA

Naive animals were anesthetized by injection of isotonic MgCl₂ (0.5 ml/g) and the two pairs of pleural-pedal ganglia were surgically removed. Each pair of ganglia was randomly assigned to either control or FMRFa group. Following trimming of the connective sheath tissue in isotonic MgCl₂:ASW (1:1 v/v), ganglia in both groups were rinsed with L15:ASW (1:1 v/v) and rested at 18 °C for 1-2 h. Subsequently, groups were treated with either vehicle (L15:ASW) or 10 μ M FMRFa for 90 min. At the end of the treatment, ganglia were rinsed with L15:ASW, rapidly frozen on dry ice, and stored at -80 °C until further processing. Total RNA was isolated from frozen pleural ganglia by addition of 750 μ l Trizol (Invitrogen) and treated with RNase-free DNase I to remove any contaminating genomic DNA. The quantification of the mRNAs was done by QRT-PCR, conducted in the Quantitative Genomics Core Laboratory of the Department of Integrative Biology and Pharmacology, The University of Texas Medical School at Houston, using a 7700 Sequence Detector (Applied Biosystems, CA) and following published procedures (Wall et al., 2003). Specific assays for ap-uch mRNAs, and for 18S rRNA, were developed with Primer Express software (Applied Biosystems). 6-carboxyfluorescein (FAM), 6-carboxy-tetramethylrhodamine (TAMRA) and the following primers and synthetic DNAs (sDNAs) were used: 18S rRNA: GenBank Accession number X94268 (Winnepenninckx et al., 1998): [1047]-CGATGCCAACTAGCGATCC-[1065], [1115]-CACTTTGGTTTCCCGGA AG-[1097], sDNA 18S rRNA:

CGATGCCAACTAGCGATCCGCAGGAGTTGCTTTGATG
 ACTCTGCGGGCAGCTTCCGGGAAACCAAAGTG; *ap-uch*: GenBank Accession number
 U90177 (Hegde et al., 1997): [587]-GAAAGGAAGCACCTGTTGTCC-[607], [656]-
 ACAACTTCAGCAGCGTCCTC-[637]; sDNA-*ap-uch*:
 GGATGGGAGAAAGGAAGCACCTGTTGTCCATGGAACGACCTCAGCAGACACAT
 TTCTTGAGGACGCTGCTGAAGTTGT.

Western blot analysis

Proteins were extracted in lysis buffer [25 mM Tris pH 7.5, 1 mM EDTA, 1% Triton X-100, 1% protease inhibitor cocktail (Sigma, St. Louis, MO), 1% phosphatase inhibitor cocktail 1 and 2 (also from Sigma)] at the times indicated after FMRFa treatment. Equal protein amount from each sample was resolved by SDS-PAGE and transferred to nitrocellulose membrane. For analysis of ubiquitination levels in pleural-pedal ganglia, membranes were incubated with an anti-ubiquitin monoclonal antibody (P4D1; Cell Signaling, Danvers, MA) and immunoreactive bands were visualized by enhanced chemiluminescence (ECL; AP Biotech, Piscataway, NJ). Signals were analyzed with ImageQuant (Molecular Dynamics, Sunnyvale CA). For analysis of *ap-uch* levels, membranes were incubated with anti-*ap-uch* antibody (kindly provided by Dr. Ashok Hegde, Wake Forest University). A polyclonal antibody against GAPDH (glyceraldehyde 3-phosphate dehydrogenase; Abcam, Cambridge, MA) was also used for normalization purposes. For the two experiments that examined the role of p38 MAPK in the upregulation of *ap-uch*, protein was isolated from Trizol-extracted samples used for mRNA quantification, as follows: after removal of the RNA-containing top phase and the interphase, 150 μ l 100% ethanol was added to the phenol layer. Following centrifugation at $12,000 \times g$ for 20 min, 750 μ l isopropanol was added to the supernatant and incubated at room temperature for 15 min. Subsequent centrifugation resulted in formation of a protein-containing pellet. The pellet was washed twice with ethanol, air-dried and solubilized in 100 μ l 0.1M NaOH. pH was neutralized with 5 μ l 20% HCl and 10 μ l 1M Tris pH 6.8. After addition of loading buffer, 40 μ l of each sample was subjected to gel electrophoresis. Subsequent analysis followed procedures described above.

Immunofluorescence analysis

Sensory neurons from pleural ganglia were cultured for 4-5 days as described previously (Angers et al., 2002). Neurons were fixed with 4 % paraformaldehyde in phosphate buffer solution (PBS) containing 30% sucrose, rinsed with PBS, and blocked for 30 min at room temperature in Superblock blocking buffer (Pierce, Holmdel, NJ) supplemented with 0.2% Triton X-100 and 3% normal goat serum. Overnight incubation with rabbit polyclonal anti-phospho-CREB2 antibody (primary) at 4° C was followed by PBS rinses and 1 h incubation with secondary antibody (goat anti-rabbit secondary antibody conjugated to Cy-3, Jackson ImmunoResearch Labs, West Grove, PA) at room temperature. For the experiments examining the stability of injected anti-phospho-CREB2 antibody, no primary antibody was added. Neurons were mounted on slides using Prolong anti-fade medium (Invitrogen, Carlsbad, CA). Images were obtained with a Bio-Rad 1024 MP confocal microscope using a 60 \times oil immersion lens. A z-series of optical sections through the cell body (0.5 μ m increments) were taken, and the section through the middle of the nucleus was used for analysis of mean fluorescence intensity with the Metamorph Offline software (Universal Imaging Corporation).

Synaptosomal preparation

Synaptosomes from 6 *Aplysia* pleural-pedal ganglia per group were prepared as described (Chin et al., 1989; Levenson et al., 2000). The synaptosomal pellet (P3) was resuspended in 1% SDS-10 mM Tris, pH 7.5, boiled and centrifuged at 14,000 rpm. The cellular fractions (approx. 1 ml each) were concentrated to approx. 100 μ l using Centricon centrifugal filter units

(Millipore, Billerica, MA). After addition of SDS and Tris (pH 7.5) to a final concentration of 1% SDS and 10 mM Tris, samples were boiled and centrifuged at 14,000 rpm. Proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes and probed with anti-synapsin, anti-synaptobrevin/VAMP (kindly provided by Dr. K.C. Martin, UCLA) and anti-GAPDH antibodies, as described in Angers et al. (2002) and above. For the experiments with the proteasome inhibitor, *clasto*-lactacystin β -lactone (50 μ M; Calbiochem, San Diego, CA) was added to the ganglia 2 h prior to and during FMRFa treatment. After FMRFa washout, the inhibitor was re-added to the bath until lysis. β -Lactone was used in this experiment because it has previously been proven effective in blocking the proteasome in *Aplysia* ganglia (Upadhyay et al., 2004). The effectiveness of lactacystin to block the proteasome has been documented in *Aplysia* cultures (e.g., Zhao et al., 2003) but not in ganglia.

Chromatin immunoprecipitation (ChIP) assay

The technique of chromatin immunoprecipitation is used to detect the interaction of a protein to a region of DNA, through a two-step process as described previously (Weinmann et al., 2001; Mohamed et al., 2005). Briefly, pleural-pedal ganglia were isolated and treated with FMRFa or vehicle for 90 min. Immediately after the treatment, the ganglia were treated with 1% formaldehyde to cross-link proteins to DNA. The reaction was quenched by the addition of glycine (0.125 M). Following cell lysis in the presence of protease inhibitors, nuclei were recovered by low-speed centrifugation, resuspended and sonicated to shear the genomic DNA to lengths of 0.3–1.3 kb. The lysates were then diluted with ChIP dilution buffer and a portion of the lysates was kept for Input control. Subsequently, the lysate was precleared with salmon sperm DNA/protein A-agarose beads, followed by brief centrifugation to pull down the beads. Antibodies were added to the resulting supernatant fraction and incubated overnight at 4° C with rotation. The following antibodies were used: anti-CREB1, 2 μ g; anti-CREB2, 2 μ g; anti-acetyl-histone H3 (Upstate cat. # 06-599, 5 μ g), anti-acetyl-histone H4 (Upstate, cat. # 06-866, 5 μ g); anti-HDAC5 (cat. # SC-5250, Santa Cruz Biotechnology; 5 μ g). Some samples were incubated with preimmune sera (or no antibody at all for the experiments in Fig. 4) as negative controls. Immune complexes were recovered by the addition of 60 μ l salmon sperm DNA/protein A-agarose slurry for 1 h at 4° C with rotation. The beads were washed with low- and high-salt washing buffers and 1 \times TE buffer. After washing, the immune complexes were eluted and cross-linking was reversed by addition of NaCl (0.3 M) followed by overnight incubation at 65° C. Following treatment with proteinase K, DNA was recovered by phenol/chloroform extraction and ethanol precipitation. DNA was resuspended in H₂O and a small aliquot was analyzed by PCR. The Input samples were not submitted to immunoprecipitation; rather, they were used directly in PCR to control for the amount of chromatin. The primer sequences (5' to 3' direction) used for detection of the *ap-uch* promoter region were the following: forward primer: [–785]-GCATTAAAGACTAGACTCTAGACTC; reverse primer: [–568]-AGATTCTAGATCTACCGGCAAGTAC.

Statistical analysis

Statistical tests were performed using MegaStat 9.1 and Jandel SigmaStat 2.0. Student t-tests were used for two-sample comparisons (Figs. 1, 3, 5A-B). ANOVA followed by post-hoc comparisons were used whenever more than two groups were compared (Figs. 5C and 6). p-values less than 0.05 were considered statistically significant.

RESULTS

An inhibitor of the proteasome blocks LTD

As a first step towards investigating the role of the proteasome in long-term depression in *Aplysia*, we examined the effect of lactacystin, an irreversible inhibitor of the 20S component of the proteasome (Myung et al., 2001), on FMRFa-induced depression of the sensorimotor

synapse (Fig. 1). Lactacystin is commonly used in *Aplysia* and rodents to block the proteasome, and its actions are comparable to those of other proteasome inhibitors (e.g., Chain et al., 1999b; Lopez-Salon et al., 2001; Zhao et al., 2003; Khoutorsky and Spira, 2005). To distinguish between a presynaptic vs. postsynaptic requirement for the proteasome, we injected lactacystin in the presynaptic sensory neuron and monitored the effects on synaptic strength after treatment with FMRFa. Briefly, following electrophysiological assessment of basal synaptic strength (Fig. 1A3, B3, pre), we pressure-injected lactacystin (10 μ M) or DMSO (0.1%, vehicle for lactacystin) into sensory neurons paired with L7 motor neurons. The efficiency of the injection was monitored under fluorescence through the addition of Alexa 488-dextran in the injection buffer (Fig. 1A2, B2). One to two hours later, cultures were treated with five 5-min pulses of FMRFa to induce LTD. This time point was chosen based on the protocol of Zhao et al. (2003). The extent of LTD and the effect of lactacystin on LTD were assessed 24 h later during the post-test (Fig. 1A3, B3, post). The DMSO+FMRFa group (mean \pm SEM: 66.31% \pm 4.5, n = 4) was significantly different from the lactacystin+FMRFa group (90.40% \pm 3.11, n = 3) (t_5 = 4.06, p < 0.01), indicating that lactacystin blocked FMRFa-induced depression (Fig. 1C). In a separate set of experiments, we examined whether lactacystin affected basal transmission over 24 h. The lactacystin-injected group (96.24% \pm 9.07, n = 4) did not differ significantly from the DMSO-injected group (87.81% \pm 8.51, n = 3) (t_5 = 0.65, p = 0.54), suggesting that lactacystin did not affect synaptic strength over a 24-h period (Fig. 1F). These results provide the first evidence that presynaptic proteasomal function is necessary for LTD in *Aplysia*.

Blockage of LTD by the inhibitor might have resulted from interfering with the initial signaling cascade activated by FMRFa (e.g., by affecting the basal turnover of proteins involved in this cascade, rather than LTD mechanisms). We think this possibility is unlikely because in our experiments the inhibitor was applied only 1 to 2 h prior to FMRFa, therefore the time window to affect the transduction cascade through a protein turnover mechanism was limited. Moreover, we found no effect of the inhibitor on basal synaptic transmission over 24 h (Fig. 1). Finally, at present there is no evidence implicating the ubiquitin-proteasome system in short-term FMRFa-induced depression. Therefore, the block of LTD after injection of the proteasome inhibitor probably reflects a requirement of the proteasome in LTD.

FMRFa exposure increased protein ubiquitination

An important step in the proteasome-mediated degradation of proteins is the attachment of ubiquitin, which serves as a tag for subsequent degradation (but see Conaway et al., 2002 for a review of proteasome-independent functions of ubiquitin). If the ubiquitin-proteasome system is necessary for LTD, then it should be possible to detect increased incorporation of ubiquitin to proteins. One method of detecting protein ubiquitination is through immunoblots using an anti-ubiquitin antibody (e.g., Ehlers, 2003). Following treatment of pleural-pedal ganglia with FMRFa or vehicle [artificial seawater (ASW); control], we monitored ubiquitination of proteins at various time points: immediately, 1 h, 2 h, 3 h and 5 h after treatment. In four independent experiments, we observed a reproducible increase in protein ubiquitination 1-2 h after the end of FMRFa exposure (Fig. 2A). This increase was not an artifact of unequal protein loading, as indicated by Ponceau staining of total protein (Fig. 2B). The time course of protein ubiquitination suggested that recruitment of the ubiquitin pathway by FMRFa is rapid but transient (Fig. 2A).

Ubiquitin hydrolase mRNA and protein are regulated in LTD

The transient nature of the increase in ubiquitination levels after treatment with FMRFa (Fig. 2) could be explained by either the transient regulation of a ubiquitinating enzyme or the engagement of deubiquitinating processes. Because one deubiquitinating enzyme, ubiquitin C-terminal hydrolase (ap-uch), is known to be necessary for and upregulated during serotonin-induced long-term synaptic plasticity (Hegde et al., 1997), we examined whether ap-uch is also

regulated during FMRFa-induced long-term plasticity. Pleural-pedal ganglia were exposed to FMRFa (10 μ M) or vehicle (ASW; control) for 85 min, followed by excision of the pleural ganglia either immediately or 1 h after treatment (Fig. 3A). QRT-PCR analysis of isolated RNA indicated that compared to time-matched controls, FMRFa induced a 4-fold increase in *ap-uch* mRNA immediately after the end of the 90-min treatment (mean \pm SEM, normalized to time-matched control: FMRFa: 444.73% \pm 114.74, $n = 5$, $t_4 = 3.01$, $p < 0.05$) (Fig. 3A). This upregulation was transient, with *ap-uch* mRNA returning to baseline 1 h after the end of treatment (normalized to time-matched control: FMRFa: 89.19% \pm 17.12, $n = 5$, $t_4 = 0.63$, $p = 0.56$). These results indicate that *ap-uch* mRNA is regulated by FMRFa.

Previous studies showed that p38 MAPK is necessary for FMRFa-induced synaptic plasticity (Guan et al., 2003). To test whether this kinase is also involved in the FMRFa-induced regulation of *ap-uch*, we preincubated ganglia for 30 min with SB203580, a p38 MAPK inhibitor. This treatment blocked the increase in *ap-uch* mRNA immediately after FMRFa (compared to time-matched SB group: SB+FMRFa: 159% \pm 32.91, $n = 5$, $t_4 = 1.8$, $p = 0.15$). As expected, SB203580 had no effect 1 h after the end of FMRFa treatment (compared to time-matched SB group: SB+FMRFa: 124.98% \pm 13.94, $n = 5$, $t_4 = 1.79$, $p = 0.15$). These results suggest that the upregulation of *ap-uch* mRNA by FMRFa requires the p38 MAPK signaling pathway.

We next examined whether the increased *ap-uch* mRNA was associated with an increase in *ap-uch* protein (Fig. 3B) using a polyclonal antibody against *ap-uch* in Western blots. This antibody was raised against the *Aplysia uch* and was previously characterized and used in Hegde et al. (1997). Membranes were re-blotted for GAPDH, which was used for normalization purposes. Compared to time-matched controls, FMRFa significantly increased *ap-uch* levels 1 h after the end of treatment (183.8% \pm 34.76, $n = 5$, $t = -2.9$, $p < 0.05$), but not immediately (165% \pm 39.53, $n = 5$, $t = -2.17$, $p = 0.1$), 2 h (170% \pm 42.47, $n = 5$, $t = -1.41$, $p = 0.23$) or 5 h (123% \pm 12.37, $n = 5$, $t = -1.44$, $p = 0.22$) after the end of treatment, although a moderate increase is evident at these time points. In two independent experiments, inhibiting p38 MAPK with SB203580 blocked the *ap-uch* increase 1 h after FMRFa [normalized to SB group: SB +FMRFa: 81.2% \pm 21.8]. These findings identify *ap-uch* as a target for regulation by FMRFa and suggest that proteolytic degradation through the ubiquitin pathway may play a role in LTD. Moreover, these results support the role of p38 MAPK as an important mediator of the actions of FMRFa.

CREB2, but not CREB1, binds to the promoter region of *ap-uch* following LTD induction

The *ap-uch* promoter region was recently cloned and a variant CRE was identified (Mohamed et al., 2005), raising the possibility that members of the CREB family of transcription factors may be involved in the regulation of *ap-uch*. Mohamed et al. (2005) investigated the interaction of CREB1 and CREB2 with the *ap-uch* promoter region under basal conditions and after treatment with 5-HT. Neither transcription factor bound to the promoter region. However, it remains possible that CREB1 or CREB2 may bind to the *ap-uch* promoter region under different conditions (e.g., after treatment with FMRFa). To explore this possibility, we performed chromatin immunoprecipitation (ChIP) assays on samples from vehicle- and FMRFa-treated ganglia using anti-CREB1 and anti-CREB2 antibodies. In three independent experiments, no basal binding of CREB1 or CREB2 to the *ap-uch* promoter region was detected, as indicated by the lack of PCR product in control groups (Fig. 4A1, A2, left lane). Moreover, treatment with FMRFa did not induce detectable binding of CREB1 (Fig. 4A1, right lane), suggesting that binding of CREB1 to the promoter region of *ap-uch* is not regulated by FMRFa. In contrast, binding of CREB2 to the promoter region was detected immediately after treatment with FMRFa (Fig. 4A2, right lane), indicating that CREB2 is recruited to the *ap-uch* promoter region during LTD induction. No changes were observed in Input controls. The

temporal dynamics of CREB2 association with the promoter region of *ap-uch* are not known. A transient association could potentially explain the transient change in *ap-uch* mRNA after FMRFa (Fig. 3). The antibodies used for the ChIP experiments are specific for CREB1 and CREB2 (Mohamed et al., 2005). Moreover, the anti-CREB1 antibodies successfully detect the association of CREB1 with the promoter region of other genes (e.g., *creb1*) (Mohamed et al., 2005), suggesting that the observed lack of CREB1 association with the *ap-uch* promoter region is not a false negative result. These findings suggest that CREB2, but not CREB1, may participate in the regulation of *ap-uch* by FMRFa.

CREB2 binding to the *ap-uch* promoter region correlates with increased histone acetylation in the *ap-uch* promoter region

The observation that CREB2 is recruited to the *ap-uch* promoter region in response to FMRFa raises the interesting possibility that CREB2 may be involved in the FMRFa-induced regulation of *ap-uch*. To further investigate this hypothesis, we examined the acetylation state of histones H3 and H4 in the promoter region of *ap-uch*. Binding of transcription factors to the promoter region of target genes recruits histone acetylases (HATs) or deacetylases (HDATs), which modify lysine residues on core histones, thus regulating the transcriptional competence of a gene. Increased acetylation by HATs correlates with transcription initiation whereas deacetylation by HDATs may lead to transcription repression (reviewed in Davie and Spencer, 1999; and Eberhardter and Becker, 2002) (but see Shahbazian and Grunstein, 2007). The acetylation state of chromatin is finely regulated during long-term synaptic plasticity and memory formation (Guan et al., 2002; Alarcon et al., 2004; Korzus et al., 2004; Levenson et al., 2004).

Using anti-acetyl-H3 or anti-acetyl-H4 antibodies in three independent ChIP experiments, some basal acetylation of H3 and H4 histones in the *ap-uch* promoter region was detected, as indicated by the presence of a PCR product in the control groups (Fig. 4B1, B2, left lane). This observation is in agreement with the constitutive expression of *ap-uch* (Hegde et al., 1997). Basal binding of histone deacetylase 5 (HDAC5) to the promoter region was also observed (Fig. 4B3, left lane), suggesting that the acetylation level of the promoter histones is dynamically regulated at rest. After treatment with FMRFa, an increase in acetyl-H3 and acetyl-H4 signals was observed compared to controls (Fig. 4B1, B2, right lane), suggesting that both histones H3 and H4 in the promoter region of *ap-uch* are hyperacetylated in response to FMRFa.

We further asked whether FMRFa signaling modulates the activity of histone deacetylases that may, in turn, alter acetylation of histones associated with the *ap-uch* promoter. Histone deacetylase 5 (HDAC5) in particular is known to be modulated by FMRFa (Guan et al., 2002). We found that recruitment of HDAC5 to the *ap-uch* promoter region was impaired (Fig. 4B3, right lane), indicating that the deacetylation of the promoter region is reduced. No changes were observed in Input controls. These FMRFa-induced changes in acetylation levels are consistent with the observation that *ap-uch* is actively transcribed during LTD, as indicated by the increase in mRNA levels (Fig. 3A). More importantly, the hyperacetylation of the promoter region occurs at the same time and in response to the same treatment that induces CREB2 binding to the promoter region, raising the possibility that CREB2 is recruited by FMRFa to activate transcription of *ap-uch*.

CREB2 is phosphorylated at a putative MAPK site during LTD induction and is necessary for LTD

The results from the ChIP experiments point to CREB2 as an important mediator of the effects of FMRFa on gene expression. If CREB2 is a key player in regulating gene expression during LTD, then CREB2 should be phosphorylated, and activated, in response to FMRFa. *In vitro*

kinase assays suggested that CREB2 can be phosphorylated by Erk and p38 MAPK (Bartsch et al., 1995; Guan et al., 2002). However, the phosphorylation of CREB2 *in vivo* has not been previously demonstrated.

To examine the potential of FMRFa to induce phosphorylation of CREB2 at a putative MAPK site, we used a phospho-specific anti-CREB2 antibody, which detects CREB2 phosphorylated at either one of two putative MAPK sites (Mohamed et al., 2005). The specificity of the antibody has been demonstrated in Western blots of membranes treated with lambda protein phosphatase (Mohamed et al., 2005), as well as in fixed cultured sensory neurons through antigen pre-absorption experiments or intracellular injection of lambda protein phosphatase (Supplementary Fig. 1)(see also Materials and Methods). Using this antibody we found that immediately after the end of treatment with FMRFa, levels of phosphorylated CREB2 (pCREB2) were significantly increased compared to control samples (FMRFa: 174.97 ± 31.58 , $n = 4$, $t = -3.44$, $p < 0.05$) (Fig. 5A). Exposure to FMRFa did not affect levels of total CREB2 (tCREB2). The increase in pCREB2 following FMRFa exposure was further observed in cultured isolated sensory neurons (Fig. 5B). Compared to controls, FMRFa treatment increased levels of pCREB2 in both the nucleus (in arbitrary units: Control: 46.42 ± 1.52 , FMRFa: 90.38 ± 5.85 , $t_{13} = -6.83$, $p < 0.001$) and the cytoplasm (Control: 48.79 ± 1.57 , FMRFa: 100.26 ± 4.96 , $t_{13} = -9.32$, $p < 0.001$) of sensory neurons, suggesting that a postsynaptic target is not required for CREB2 to be phosphorylated in response to FMRFa. Furthermore, these results show that FMRFa can have the same long-term biochemical effects on cells in culture and in ganglia.

We further hypothesized that blocking phosphorylated CREB2 would impair LTD. To investigate the role of phosphorylated CREB2 in LTD, we injected presynaptic sensory neurons co-cultured with motor neurons with the same anti-phospho-CREB2 antibody that we used for our Western blots and immunofluorescence experiments, and we monitored the effects on LTD expression (Fig. 5C). The anti-phospho-CREB2 antibody was expected to functionally sequester phospho-CREB2 in the cytoplasm, preventing its nuclear translocation and blocking pCREB2-dependent functions. Indeed, our immunofluorescence experiments showed that phosphorylated CREB2 localizes in both the nucleus and the cell body (Fig. 5B), as observed in rat cultured cortical neurons (White et al., 2000). Moreover, Lai et al. (2006) showed that following treatment with FMRFa, CREB2 translocates to the nucleus in an importin-dependent manner. The antibody was not expected to enter the nucleus due to size exclusion (Ohno et al., 1998).

The specificity of the anti-phospho-CREB2 antibody, which was also used in Western blots and immunofluorescence experiments (Fig. 5A,B), is demonstrated by the following: i) BLAST searches indicated that the peptide sequence of *Aplysia* CREB2 against which the antibody was raised, is not contained in any other known *Aplysia* protein; ii) the antibody was affinity-purified twice by passing through a phospho-CREB2 column and a total CREB2 column (Mohamed et al., 2005); iii) in Western blots, the antibody specifically immunoreacted with a single band of the expected molecular mass (50 kDa)(Fig. 5A); iv) preabsorption of the antibody with a CREB2 phospho-peptide blocked immunoreactivity in cultured sensory neurons (Fig. S1 A1-2), whereas preabsorption with the unphosphorylated version of the same peptide did not (Fig. S1 A3-4); v) antibody immunoreactivity was dramatically reduced in sensory neurons after dephosphorylation of endogenous CREB2 by injecting a protein phosphatase (Fig. S1C); vi) brief treatment of membranes with protein phosphatase impaired immunoreactivity of the anti-phospho-CREB2 antibody in Western blots of *Aplysia* protein extracts (Mohamed et al., 2005).

After assessment of synaptic strength (Fig. 5C1; pre), we injected anti-phospho-CREB2 (anti-pCREB2) antibody or purified rabbit IgG into sensory neurons. Following a rest period of 1

h, co-cultures were exposed to five, 5-min pulses of FMRFa or vehicle (ASW) at an inter-pulse interval of 20 min. Synaptic strength was reassessed 24 h later (Fig. 5C1; post) to evaluate the extent of LTD. Two-way ANOVA indicated that there was a significant interaction between factors [the injected antibodies (anti-pCREB2 or rabbit IgG) and the treatment (FMRFa or vehicle)] ($F_{1,28} = 8.013$, $p < 0.05$). Post-hoc comparisons revealed that in the IgG-injected group, FMRFa induced significant depression compared to vehicle-treated controls (IgG + FMRFa: $79.43\% \pm 4.58$, IgG: $100.5\% \pm 6.2$, $q = 4.00$, $p < 0.05$). However, in the anti-pCREB2 group FMRFa failed to induce significant depression (pCREB2: $86.5\% \pm 7.3$, pCREB2 + FMRFa: $92.97\% \pm 5.63$, $q = 1.464$, $p > 0.05$). Importantly, a significant difference was found between the anti-pCREB2+FMRFa group and the IgG+FMRFa group ($q = 3.04$, $p < 0.05$), indicating that injection of anti-pCREB2 antibody blocked FMRFa-induced LTD (Fig. 5C2). Injection of IgG or anti-pCREB2 antibody alone did not significantly impair transmission over the 24-h period ($q = 2.67$, $p > 0.05$). Collectively, these results suggest that CREB2 is phosphorylated in LTD and that pCREB2 is necessary for LTD.

One concern about the antibody injection experiment was whether the anti-CREB2 antibody would be stable in the cellular milieu so as to functionally block endogenous CREB2. Our finding that anti-pCREB2 injection blocked LTD would argue for the stability of the antibody. Nonetheless, we performed a control experiment to examine the presence of the antibody within the cell over time. To this end, we injected the anti-pCREB2 antibody in sensory neurons and then fixed them either 2 h or 24 h post injection. Cells were incubated only with secondary antibody (to detect levels of injected primary antibody) and processed for immunofluorescence. pCREB2 immunofluorescence could be detected both at 2 h and 24 h post injection (pCREB2 signal normalized to injection dye: 2 h: 0.97 ± 0.06 , 24 h: 0.92 ± 0.04). The small decrease in signal intensity that was observed at 24 h compared to 2 h was not statistically significant ($t_{17} = 0.69$, $p = 0.50$). These results indicate that the pCREB2 antibody is stable *in vivo* for at least 24 h.

Levels of synapsin are decreased in synaptosomes during LTD

Results from the experiments discussed above point to the ubiquitin-proteasome system as a necessary mechanism for LTD, supporting a role of regulated protein degradation in the induction and/or consolidation of LTD. Previous work also showed that FMRFa-induced LTD of sensorimotor synapses is accompanied by a significant loss of sensory neuron varicosities and neurites (Schacher and Montarolo, 1991), raising the possibility that synaptic proteins are targeted for degradation during LTD. Synapsin is a protein that has been implicated not only in the regulation of synaptic strength (Llinas et al., 1985; Hilfiker et al., 1998) and plasticity (Humeau et al., 2001; Chi et al., 2003; Fioravante et al., 2007) but also in synapse formation and maintenance as well as neurite elongation (Ferreira and Rapoport, 2002). The *Aplysia* homologue of synapsin was cloned and found to localize primarily at varicosities (Angers et al., 2002). To begin to investigate mechanisms of LTD expression, we tested whether synapsin is degraded during LTD. Twenty four hours after treatment with FMRFa or vehicle (ASW; control), we prepared synaptosomes from pleural-pedal ganglia and examined levels of synapsin using Western blot analysis. Results from 4 independent experiments indicated that significantly less synapsin was present in synaptosomes from ganglia treated with FMRFa compared to controls (synapsin: $64\% \pm 9.19$, $t_3 = 3.33$, $p < 0.05$).

To begin to examine the mechanisms underlying synapsin degradation, we repeated the above experiment with or without the proteasome inhibitor clasto-lactacystin β -lactone (Fig. 6A). This irreversible inhibitor of the proteasome is a derivative of lactacystin that effectively blocks protein degradation in *Aplysia* ganglia (Upadhyaya et al., 2004) and cultures (Zhao et al., 2003; Khoutorsky and Spira, 2005). In three independent experiments, we replicated our previous finding that FMRFa decreased levels of synaptosomal synapsin 24 h after treatment

(mean \pm SEM expressed as % control: FMRFa: 21.17 ± 6.94) (Fig. 6A). Preincubation with β -lactone at 50 μ M, a concentration used by Upadhyaya et al. (2004), blocked this decrease (β -Lactone+FMRFa: 96.08 ± 2.37). One way ANOVA showed a significant difference between the 4 groups [DMSO (vehicle for β -Lactone), DMSO+FMRFa, β -Lactone, β -Lactone+FMRFa: $F_{3,11} = 7.79$, $p < 0.01$]. Subsequent post-hoc comparisons indicated that the β -Lactone+FMRFa group differed significantly from the FMRFa group ($q = 2.65$, $p < 0.05$) but not from the β -Lactone group ($q = 0.16$, $p > 0.05$), or the DMSO group ($q = 2.16$, $p > 0.05$), suggesting that the inhibitor blocked the FMRFa-induced decrease in synapsin. This effect of FMRFa targeted synapsin specifically and probably did not reflect loss of synaptic vesicles because levels of the synaptic vesicle marker synaptobrevin/VAMP were not affected by FMRFa treatment (FMRFa: $92.68\% \pm 19.4$) (Fig. 6A). Finally, the decrease in synapsin was not observed in the cellular fraction (FMRFa: $108.11\% \pm 9.88$) (Fig. 6B), suggesting that the synaptosomal decrease in synapsin levels results from protein degradation and not subcellular redistribution. A decrease in synapsin may or may not be evident in total lysates, depending on whether synapsin degradation is a predominantly synaptic event. The 8% increase in the cellular fraction is within the margin of statistical error. Our interpretation of these results is that during the first 24 h after FMRFa treatment, synapsin is downregulated at synaptic sites, probably through the action of synaptically localized proteasomes (Bingol and Schuman, 2005; Upadhyaya et al., 2006), but not in other cellular compartments.

DISCUSSION

Using the *Aplysia* model system, we sought to identify mechanisms mediating a form of long-term heterosynaptic depression induced by the neuropeptide FMRFa. For our studies we used both the *Aplysia* sensorimotor synapse, which is a well studied model for the investigation of synaptic plasticity (Kandel, 2001), as well as whole pleural-pedal ganglia. The approach of using both whole ganglia and cultured sensory neurons in a complimentary way to investigate mechanisms of synaptic plasticity has been used successfully in the work of others (Guan et al., 2002; Guan et al., 2003; Si et al., 2003; Udo et al., 2005; Lee et al., 2007), yielding significant findings.

Since its discovery in bivalves (Price and Greenberg, 1977), an extensive family of FMRFa-related peptides (FaRPs) has been described in *Drosophila* (reviewed by Taghert, 1999), *C. elegans* (reviewed by Li et al., 1999), and rodents (reviewed by Raffa, 1990). However, the function of FaRPs in these systems is largely unknown. In *Aplysia*, FMRFa-containing neurons are wide spread (Brown et al., 1985; Lloyd et al., 1987; Xu et al., 1994) and activation of some of them produces presynaptic inhibition of sensory neuron synapses (Mackey et al., 1987; Small et al., 1992). FMRFa induces both short- and long-term synaptic depression. Short-term depression, which has been observed in both cultures and ganglia (Pieroni and Byrne, 1992; Guan et al., 2003), emerges at least in part from modulation of ionic conductances and the release machinery (Belardetti et al., 1987; Piomelli et al., 1987; Dale and Kandel, 1990; Critz et al., 1991). Long-term depression, which at present has only been examined in cultures, requires gene expression (Montarolo et al., 1988; Bailey et al., 1992). However, the identities of LTD-related genes and of the transcription factors that regulate them are largely unexplored. The present study provides the first evidence that a proteasome-associated ubiquitin hydrolase is upregulated in LTD and identifies CREB2 as a potential mediator of this regulation.

Using a pharmacological inhibitor, we found that the ubiquitin-proteasome system is necessary for LTD. Other evidence from *Aplysia* and mammals also points to the role of this system in synaptic plasticity and learning. Specifically, the proteasome is involved in long-term synaptic facilitation (LTF) (Chain et al., 1999b; Zhao et al., 2003). In LTF, the regulatory subunit of protein kinase A (PKA) is degraded, giving rise to persistently active catalytic subunits. Long-term PKA activation could prolong phosphorylation of transcription activators, such as CREB1

(Chain et al., 1999b; Liu et al., 2008). Moreover, the transcription inhibitor CREB1b is targeted for degradation during LTF (Upadhyay et al., 2004). Together, these molecular events could lead to increased expression of genes essential for consolidation of LTF. Also, in rodents the ubiquitin pathway seems to be important in memory formation because bilateral infusion of a proteasome inhibitor in the hippocampus impaired inhibitory avoidance learning (Lopez-Salon et al., 2001) and extinction of fear conditioning (Lee et al., 2008). Furthermore, long-term potentiation (LTP) and context-dependent learning were impaired in mice carrying a mutation in E6-AP ubiquitin ligase, an enzyme involved in the ubiquitin pathway (Jiang et al., 1998). Recently, LTP induction was reported to accelerate protein degradation via the proteasome system (Karpova et al., 2006) (but see Dong et al., 2008). Finally, the proteasome and protein ubiquitination are important for AMPA receptor internalization and LTD in the hippocampus (Colledge et al., 2003).

Following exposure to FMRFa, we observed a rapid, yet transient, increase in protein ubiquitination. Even if ubiquitination persisted for 24 h, a causal link between ubiquitination and LTD could not be claimed without further analysis. Nonetheless, it seems likely that the proteasome is involved in LTD based on the following findings: i) ubiquitination is increased in response to FMRFa treatment; ii) blocking the proteasome by injecting lactacystin blocks LTD; and iii) lactacystin blocks the FMRFa-induced downregulation of synapsin. Increased protein ubiquitination has also been observed in cortical neurons following pharmacologically-induced changes in synaptic activity (Ehlers, 2003).

The duration and extent of protein ubiquitination is partly controlled by a large family of deubiquitinating enzymes (DUBs), which are involved in removing and recycling ubiquitin from ubiquitin-tagged proteins (Glickman and Ciechanover, 2002). Therefore, we examined the potential involvement of DUBs in LTD. In *Aplysia*, one DUB is ubiquitin hydrolase (*ap-uch*), a neuron-specific hydrolase that is necessary for LTF and is upregulated by 5-HT (Hegde et al., 1997). This upregulation appears to be independent of CREB1 and CREB2 (Mohamed et al., 2005). In our study, we found that *ap-uch* is upregulated early during LTD in a p38 MAPK-dependent manner, suggesting that *ap-uch* may participate in regulating the duration of ubiquitination. If *ap-uch* is involved in both LTF and LTD, how is the direction of synaptic plasticity (facilitation vs. depression) attained? One possible explanation is that increased protein degradation is important for both forms of synaptic plasticity, and that the direction of plasticity at least partly derives from specific combinations of ubiquitin conjugating and ubiquitin ligase enzymes, which would target particular proteins for degradation (Cline, 2003; Yi and Ehlers, 2005). Such target proteins may include the regulatory subunit of PKA, whose dissociation from the catalytic subunit is triggered by 5-HT and seems to be required for its degradation by the proteasome (Chain et al., 1999a).

In our experiments, CREB2 bound to the *ap-uch* promoter in response to FMRFa, but not under basal conditions, and this binding correlated with histone hyperacetylation, suggesting that the *ap-uch* promoter becomes transcriptionally active under the control of CREB2. Indeed, CREB2 is known to induce gene expression in F9 cells (Bartsch et al., 1995). Moreover, mammalian CREB2, which shows significant homology to *Aplysia* CREB2, can function as an activator of transcription (Siu et al., 2002; Luo et al., 2003) even though it is traditionally regarded as a repressor (Karpinski et al., 1992). Furthermore, mammalian CREB2 possesses a strong transcription activator domain but no repressor domain (Schoch et al., 2001; Thiel et al., 2005), suggesting that CREB2 may act as an activator of transcription under certain conditions.

We also found that FMRFa induced phosphorylation of CREB2 at a putative MAPK site. Furthermore, in agreement with previous observations that blocking total CREB2 impaired LTD (Guan et al., 2002), intracellular injections of anti-phospho-CREB2 antibody blocked LTD. However, one possibility is that blockage of LTD arises from interfering with the initial

signaling cascade activated by FMRFa. We believe that this possibility is unlikely because in contrast to long-term depression, short-term depression does not depend on protein synthesis (Montarolo et al., 1988; Bailey et al., 1992). Moreover, in our hands (Fig. 5) as well as in experiments of others (Bartsch et al., 1995; Guan et al., 2002), injection of anti-CREB2 antibodies does not significantly affect basal synaptic transmission. Therefore, the block of LTD after injection of anti-phospho-CREB2 antibodies probably reflects a requirement of phospho-CREB2 in LTD.

In previous studies CREB2 was assigned the role of transcription repressor because binding of CREB2 to the promoter of the gene *c/ebp* (CCAAT enhancer binding protein), which is upregulated by 5-HT and is necessary for LTF (Alberini et al., 1994), recruited HDAT5 and correlated with reduced C/EBP mRNA levels. Our results confirm the essential role of CREB2 in LTD and provide additional information on its phosphorylation state after FMRFa exposure. p38 MAPK probably mediates the FMRFa-induced phosphorylation of CREB2 *in vivo* because p38 MAPK is activated by FMRFa, is necessary for LTD, and can phosphorylate CREB2 *in vitro* (Guan et al., 2003). Moreover, our results showed that pretreatment with a p38 MAPK inhibitor blocked the FMRFa-induced regulation of *ap-uch*. Finally, based on our ChIP assays, we propose a role for CREB2 as transcription activator and we identify *ap-uch* as one candidate gene whose regulation may be mediated by CREB2.

Recent studies revealed that, in parallel to long-term potentiation/facilitation (Bailey and Kandel, 1993; Yuste and Bonhoeffer, 2001), LTD protocols produce persistent morphological changes. In hippocampal slices, low frequency stimulation, which gives rise to LTD, induces spine retraction (Nagerl et al., 2004) and shrinkage (Zhou et al., 2004). In *Aplysia*, LTD in sensorimotor co-cultures is accompanied by reduction in the number of presynaptic varicosities and retraction of processes (Schacher and Montarolo, 1991). Nonetheless, specific target proteins associated with these structural changes have not been identified. The synaptic vesicle protein synapsin, apart from its role in vesicle regulation (Hilfiker et al., 1999), is known to play a role in synapse formation and neurite elongation (Chin et al., 1995; Ferreira et al., 1995; Kao et al., 2002). We now provide evidence that synapsin is a target for regulated protein degradation during LTD. Levels of synapsin are downregulated in synaptosomes 24 h after LTD induction. This downregulation depends on the proteasome and does not seem to reflect loss of synaptic vesicles because levels of another vesicle protein, VAMP, are not affected by FMRFa at least during the first 24 hours after treatment. Because our synaptosomal preparations were obtained from pleural-pedal ganglia, which include sensory neurons as well as other types of cells, an alternative explanation for the differential effect of FMRFa on synapsin and VAMP could be that the two proteins are differentially regulated in various cell types. Another possibility is that the time course of varicosity elimination differs in cultures and ganglia. In cultures, FMRFa-induced varicosity elimination and retraction of neurites occurs within 24 h (Schacher and Montarolo, 1991), whereas these phenomena have not been characterized in ganglia. We propose that regulation of synapsin may be important for LTD, in a manner that remains to be described. Also, the temporal dynamics of synapsin regulation during LTD remain unknown. Based on our observation that total protein ubiquitination is increased early after FMRFa (Fig. 2), we would predict that synapsin downregulation should also be detectable at time points earlier than 24 h. Presynaptic proteins including syntaxin and RIM1 are known to be regulated through ubiquitination (Chin et al., 2002; Chen et al., 2003; Yao et al., 2007) but a link between proteasome-mediated degradation of presynaptic proteins and long-term plasticity has not been previously reported.

We propose a conceptual model that begins to explain some of the mechanisms involved in long-term depression. Upon FMRFa stimulation, p38 MAPK becomes activated and phosphorylates CREB2. This phosphorylation of CREB2 triggers both repression and enhancement of transcription, depending on the target genes. For example, binding of CREB2

to the promoter regions of genes such as *c/ebp* would repress transcription. In contrast, binding of phosphorylated CREB2 to the promoter regions of a different set of genes (probably with different response elements than *c/ebp*) including *ap-uch*, would enhance transcription. Protein ubiquitination is increased in response to FMRFa, tagging proteins for degradation. Increased levels of ap-uch would help enhance degradation of ubiquitinated proteins, which is an essential step in the induction of LTD. One such protein could be synapsin, the degradation of which may be important for downregulating synaptic strength and structures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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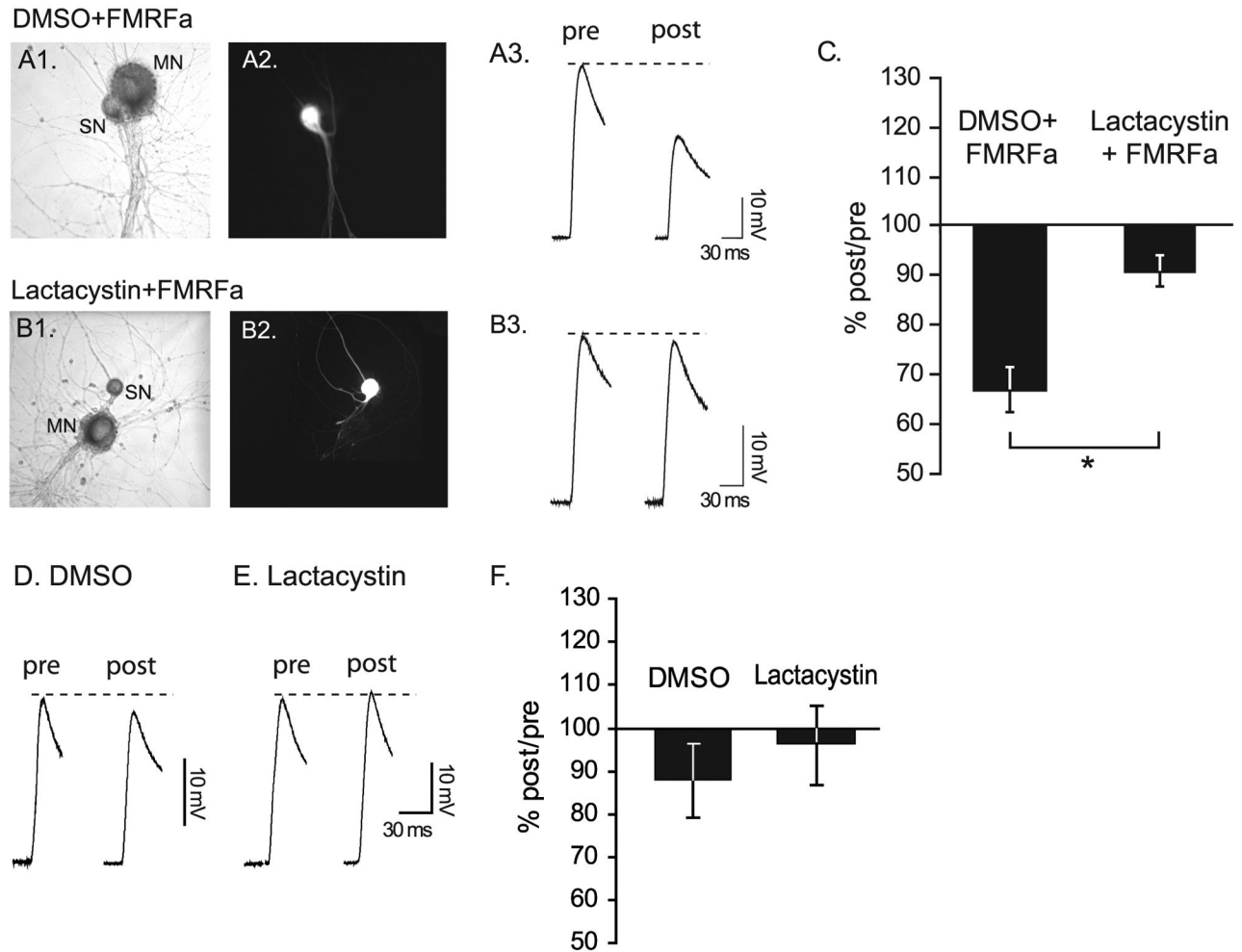
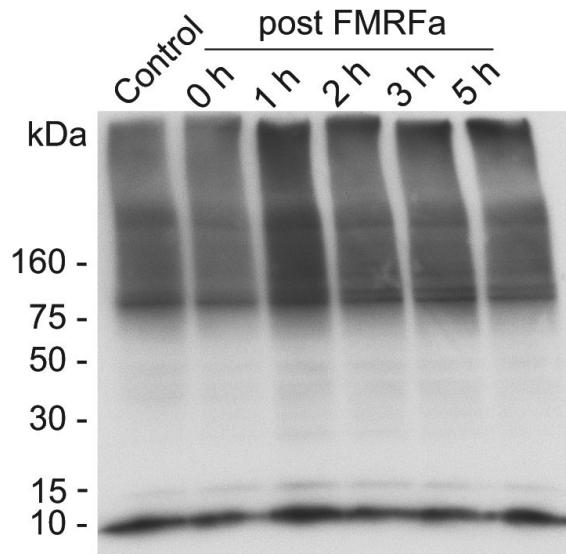


Figure 1.

Lactacystin blocked FMRFa-induced LTD of sensorimotor synapse. A1, B1, Bright-field images of sensorimotor co-cultures. A2, B2, Sensory neurons filled with Alexa 488-dextran, indicating successful injection of DMSO (A2) or lactacystin (B2). A3, B3, Examples of excitatory postsynaptic potentials (EPSPs) measured before the presynaptic injection (pre) and 24 h after treatment with FMRFa (post). EPSPs were elicited by extracellular stimulation of the sensory neuron. C, For statistical analysis, the ratio of post over pre EPSP amplitude was formed for each co-culture. * $p < 0.01$. D, E, Examples of EPSPs measured before (pre) and approx. 24 h after (post) presynaptic injection of DMSO (vehicle) or lactacystin. F, Lactacystin did not significantly affect synaptic strength over a 24-h period ($p = 0.54$).

A. Anti-ubiquitin antibody



B. Ponceau staining

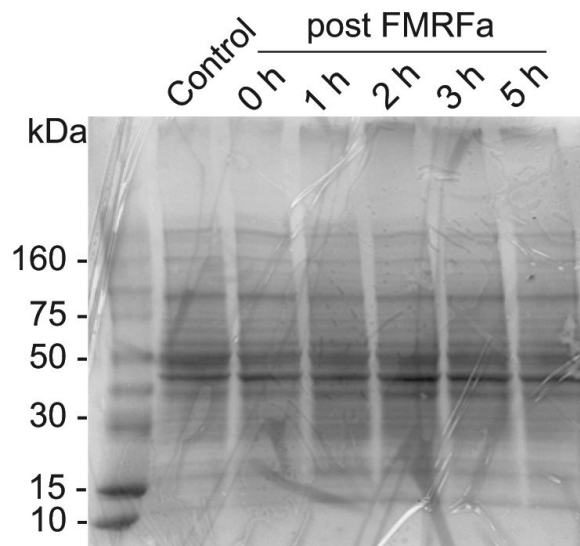


Figure 2.

Protein ubiquitination is increased 1-2 h after treatment with FMRFa. Following exposure to FMRFa or vehicle (ASW; control), pleural-pedal ganglia were lysed at various time points as indicated and processed with an anti-ubiquitin antibody to monitor levels of protein ubiquitination. Note that the 0 h time point corresponds to approx. 85 min of FMRFa exposure. A, Increased protein ubiquitination was observed 1-2 h after the end of FMRFa exposure. The 10 kDa band is most probably free ubiquitin. B, Ponceau staining of the nitrocellulose membrane confirmed equal loading of samples. Representative examples of four independent experiments.

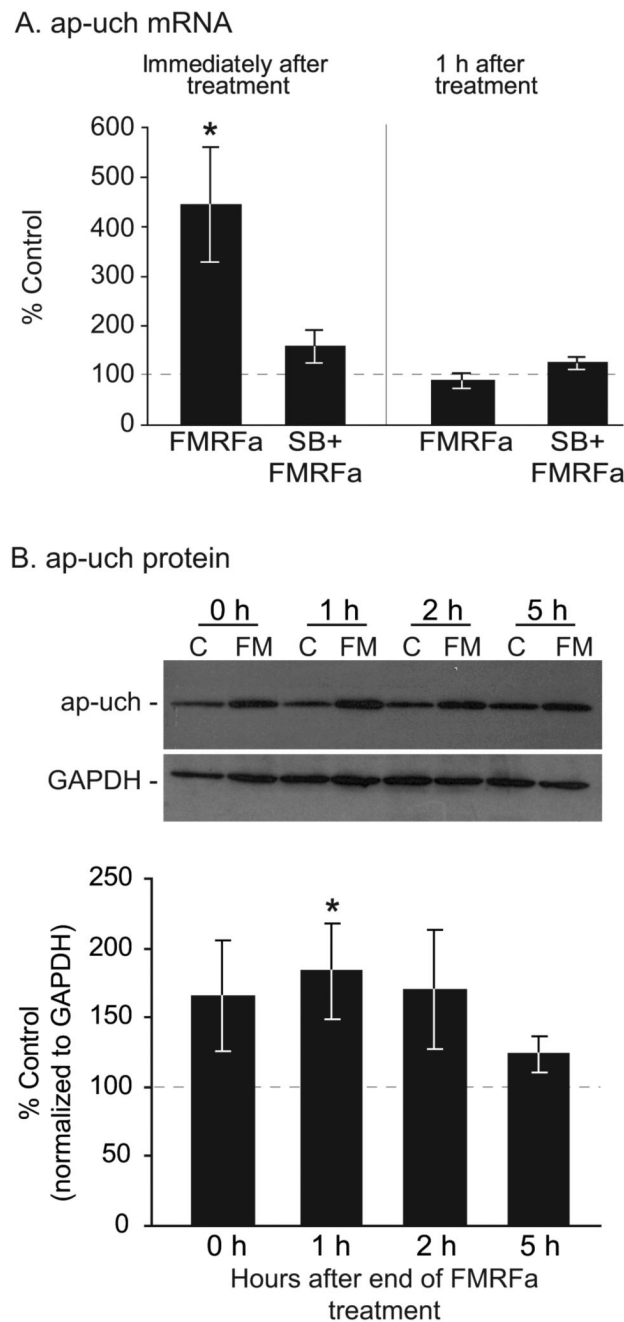


Figure 3.

FMRFa upregulates ap-uch mRNA and protein in pleural ganglia. A, QRT-PCR analysis of mRNA extracted from ganglia either immediately or 1 h after the end of 90-min treatment with FMRFa or vehicle (control). Immediately after the end of treatment, a significant increase in ap-uch mRNA was observed compared to time-matched control (* $p < 0.05$). In the presence of the p38 kinase inhibitor SB203580, FMRFa did not induce an increase in ap-uch mRNA. The effect of FMRFa on ap-uch was transient because 1 h after the end of treatment the mRNA was at control levels. B, Using an antibody specific for ap-uch, Western blot analysis was conducted on extracts from ganglia treated with FMRFa or vehicle (artificial seawater; control) and lysed immediately, 1 h, 2 h or 5 h after the end of treatment. Membranes were also probed

with an anti-GAPDH antibody for normalization purposes. Compared to time-matched controls, ap-uch protein significantly increased only at the 1 h time point, although a tendency for increase was observed at all time points examined (* $p < 0.05$). These results indicate that the upregulation of ap-uch mRNA is accompanied by increased ap-uch protein synthesis.

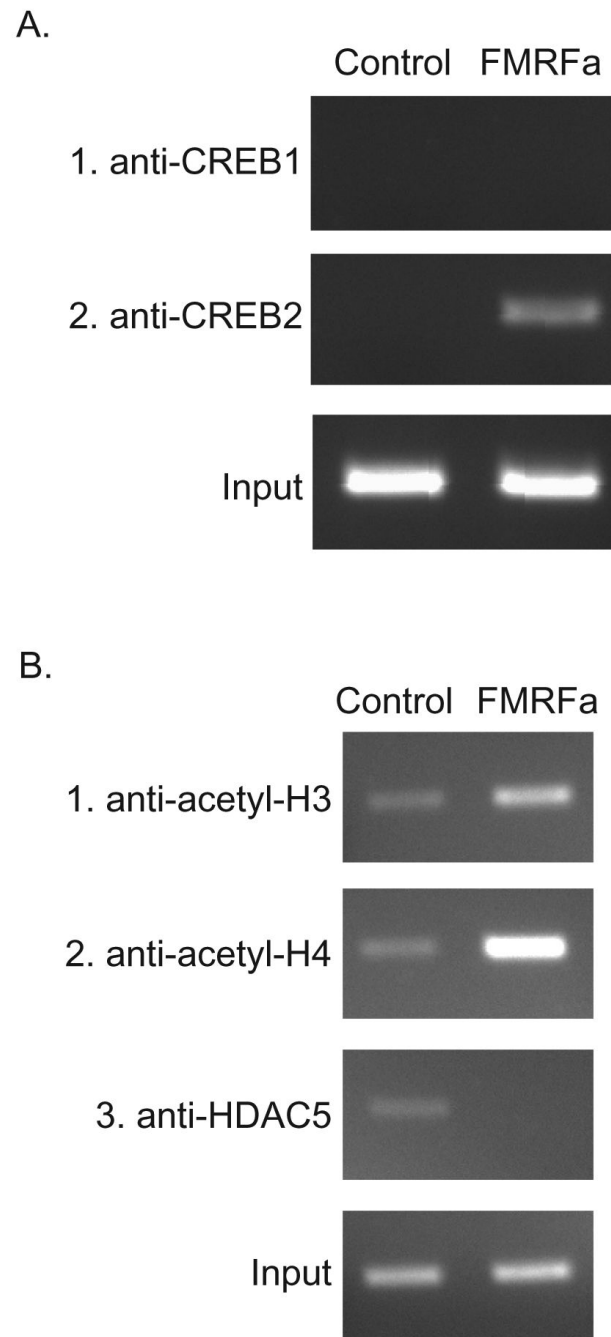
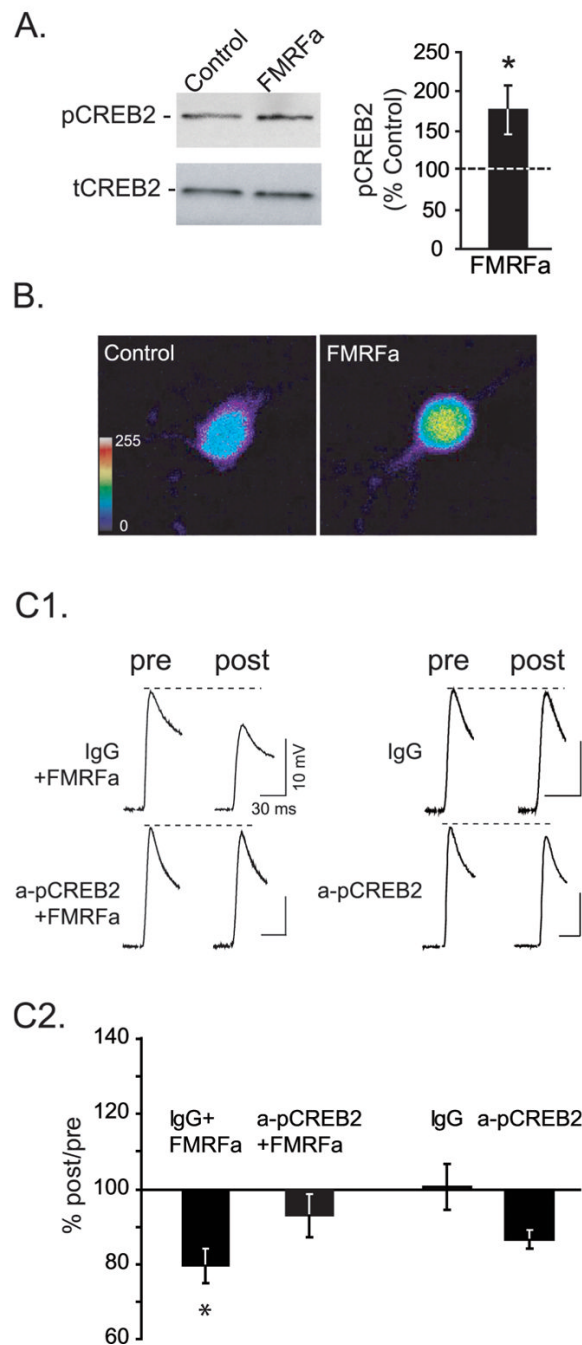


Figure 4.

Binding of CREB2, but not CREB1, to the promoter region of *ap-uch* coincides with histone hyperacetylation during LTD induction. Following treatment with FMRFa or vehicle (ASW; control), chromatin was prepared from ganglia and immunoprecipitated with the indicated antibodies. The immunoprecipitated chromatin was analyzed by PCR as described in the Methods. Chromatin samples before immunoprecipitation (Input) were also analyzed to control for amount of starting material. A, Under basal conditions, no association of CREB2 or CREB1 with the *ap-uch* promoter region was detected (A1, A2, left lane). FMRFa did not induce detectable binding of CREB1 to the promoter region (A1, right lane) but it did stimulate binding of CREB2 (A2, right lane), indicating that CREB2 is recruited to the *ap-uch* promoter region

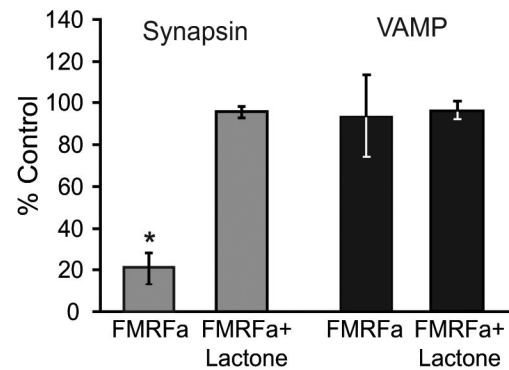
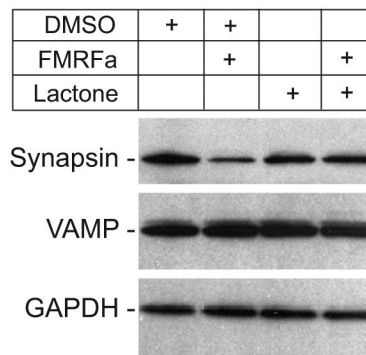
during LTD induction. No change in Input was observed. Representative examples of three independent experiments. B, The acetylation state of histones in a promoter region correlates with transcription competence: increased acetylation correlates with transcription initiation, whereas deacetylation by histone deacetylases (HDATs) may lead to transcription repression. Using anti-acetyl-H3 or anti-acetyl-H4 antibodies, basal acetylation of H3 and H4 histones was detected in the *ap-uch* promoter region (B1, B2, left lane). Basal binding of histone deacetylase 5 (HDAC5) to the promoter region was also observed (B3, left lane). FMRFa caused increased acetylation of both histones H3 and H4 (B1, B2, right lane) as well as reduced recruitment of HDAC5 at the *ap-uch* promoter region (Fig. B3, right lane), indicating that the promoter region of *ap-uch* becomes hyperacetylated, and therefore permissive to transcription, after FMRFa. No change in Input was observed. Representative examples of three independent experiments.

**Figure 5.**

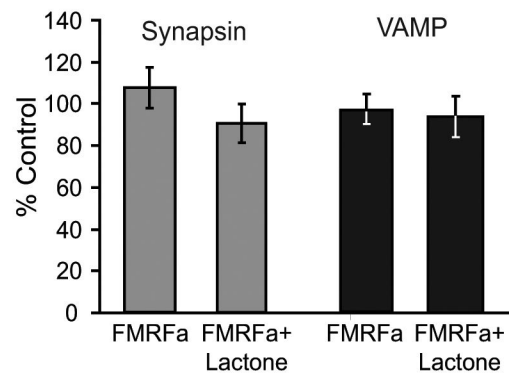
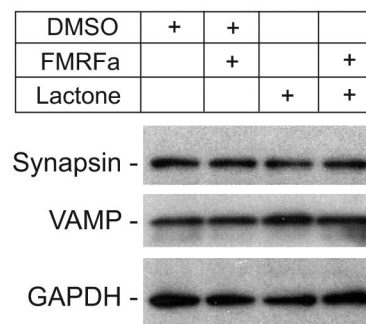
CREB2 is phosphorylated following FMRFa treatment and is necessary for FMRFa-induced LTD. A, Immunoblot analysis using the anti-phospho-CREB2 antibody showed that immediately after the end of treatment with FMRFa, levels of phosphorylated CREB2 (pCREB2) were significantly increased compared to control. Exposure to FMRFa did not affect levels of total CREB2. (* $p < 0.05$). B, Under control conditions, pCREB2 was observed primarily in the nucleus of cultured isolated sensory neurons and to a lesser extent in the cytoplasm, displaying a distribution pattern that is similar to that of mammalian CREB2 in rat cultured cortical neurons (White et al., 2000). Following FMRFa exposure, an increase in pCREB2 was observed in both the cytoplasm and nucleus, suggesting that the presence of a postsynaptic

target is not required for CREB2 to be phosphorylated. C1, Representative EPSPs recorded from IgG-injected (upper traces) or phospho-specific anti-CREB2-injected (lower traces) co-cultures, before and after treatment with FMRFa or vehicle. C2, For statistical analysis, the peak amplitude of EPSP at the 24-h post-test was normalized to pre-test EPSP. Injection of anti-phospho-CREB2 antibody blocked LTD (* $p < 0.05$) without significantly affecting basal transmission.

A. Synaptosomal fraction



B. Cellular fraction

**Figure 6.**

Synapsin is downregulated in LTD in a proteasome-dependent manner. A, Western blot analysis of synaptosomes prepared from FMRFa-treated or control ganglia. Membranes were probed with anti-synapsin, anti-VAMP and anti-GAPDH antibodies. GAPDH signal was used for normalization purposes. Results indicated that compared to untreated ganglia, synapsin was significantly decreased 24 h after FMRFa (* $p < 0.05$). This decrease was blocked by pre-incubation with the proteasome inhibitor *clasto*-lactacystin β -lactone. Levels of the synaptic vesicle associated membrane protein synaptobrevin/VAMP were unaffected by the treatment. B, Western blot analysis of the cellular fractions from the same cell fractionation experiment as in A. FMRFa treatment had no significant effect on levels of synapsin or VAMP. Note enrichment of synapsin and VAMP in the synaptosomal fraction (A) compared to the cellular fraction (B).